

# **Beetroot juice is more beneficial than sodium nitrate for attenuating muscle pain after strenuous eccentric-bias exercise**

Tom Clifford<sup>1</sup>, Glyn Howatson<sup>2,3</sup>, Daniel J. West<sup>4</sup>, Emma J. Stevenson<sup>4</sup>

<sup>1</sup> School of Biomedical Sciences, Newcastle University, United Kingdom; <sup>2</sup> Faculty of Health and Life Sciences, Department of Sport, Exercise & Rehabilitation, Northumbria University, Newcastle, United Kingdom; <sup>3</sup> Water Research Group, School of Environmental Sciences and Development, Northwest University, Potchefstroom, South Africa; <sup>4</sup> Human Nutrition Research Centre, Institute of Cellular Medicine, Newcastle University, Newcastle, United Kingdom. Emails: [tom.clifford@newcastle.ac.uk](mailto:tom.clifford@newcastle.ac.uk); [glyn.howatson@northumbria.ac.uk](mailto:glyn.howatson@northumbria.ac.uk) ; [daniel.west@newcastle.ac.uk](mailto:daniel.west@newcastle.ac.uk); [emma.stevenson@newcastle.ac.uk](mailto:emma.stevenson@newcastle.ac.uk).

## **Corresponding author:**

Tom Clifford

School of Biomedical Sciences

Newcastle University

Newcastle-upon-Tyne

NE1 7RU

UK

Tel: +44 0192 088 311

## **Abstract**

The aim of this study was to compare the effects of beetroot juice (BTJ) and a nitrate only drink (sodium nitrate; SN) on indices of exercise-induced muscle damage (EIMD). Thirty recreationally active males consumed either BTJ ( $n=10$ ), a nitrate matched SN drink ( $n=10$ ) or an isocaloric placebo (PLA;  $n=10$ ) immediately, 24 and 48 h after performing 100 drop jumps. To assess muscle damage, maximal isometric voluntary contractions (MIVC), countermovement jumps (CMJ), pressure-pain threshold (PPT), creatine kinase (CK) and high sensitivity C-reactive protein (hs-CRP) were measured pre, immediately post, 24, 48 and 72 h following the drop jumps. BTJ and SN increased serum nitric oxide, which peaked at 2 h post-ingestion ( $136\pm78$  and  $189 \pm 79$   $\mu\text{mol/L}$ , respectively). PPT decreased in all groups post-exercise ( $P = 0.001$ ), but was attenuated with BTJ compared to SN and PLA ( $P = 0.043$ ). PPT was  $104\pm26\%$  of baseline values 72 h post after BTJ;  $94\pm16\%$  after SN, and;  $91\pm19\%$  after PLA. MIVC and CMJ were reduced following exercise ( $-15$ - $25\%$ ) and did not recover to baseline by 72 h in all groups; however, no group differences were observed ( $P > 0.05$ ). Serum CK increased after exercise but no group differences were present ( $P > 0.05$ ). hsCRP levels were unaltered by the exercise protocol ( $P > 0.05$ ). These data suggest that BTJ supplementation is more effective than SN for attenuating muscle pain associated with EIMD, and that any analgesic effects are likely due to phytonutrients in BTJ other than nitrate, or interactions between them.

**Key words:** MUSCLE PAIN; MUSCLE DAMAGE; RECOVERY; BETALAINS; RECOVERY; NITRATE

## **Introduction**

We have recently shown that beetroot juice (BTJ), which has mostly been studied for its potential as a pre-exercise ergogenic aid, might also hold some promise as a recovery aid following muscle-damaging exercise (Clifford et al. 2016a,c). More specifically, we found that consuming BTJ for 3 days after either a bout of drop jumps (Clifford et al., 2016a) or a bout of repeated sprints (Clifford et al., 2016c), accelerated the recovery of counter movement jump (CMJ) performance and attenuated muscle pain. These findings do not appear to be consistent across all types of exercise, however, as BTJ did not benefit recovery after a marathon (Clifford et al., 2016d), which was likely related to the significantly smaller magnitude of muscle damage in this study.

The mechanisms by which BTJ improved exercise recovery in the aforementioned studies is unclear. Our initial hypothesis was that, akin to other functional foods shown to attenuate exercise-induced muscle damage (EIMD), such as cherry juice (Bell et al. 2014; Bell et al. 2015; Bowtell et al. 2011; Connolly et al. 2006; Howatson et al. 2010) and pomegranate juice (Trombold et al. 2010; Trombold et al. 2011), it could be through an anti-inflammatory or antioxidant (AOX) related mechanism. Nonetheless, it is equivocal as to whether beetroot juice and/or its constituents are effective AOXs, with some studies suggesting not (Whitfield et al., 2016; Larsen et al., 2014) and others showing reductions in oxidative stress and inflammatory markers (Clifford et al. 2015; El Gamal et al. 2014; Jadert et al. 2012; Justice et al. 2015; Pietrzkowski et al. 2010; Ashor et al., 2016). Notwithstanding the disparate findings to date, it is conceivable that AOX or anti-inflammatory effects could help to dampen the acute secondary muscle damage response after an exercise bout—the hallmarks of which are proposed to be oxidative stress, inflammation and muscle proteolysis—and this could facilitate a faster recovery of muscle function and/or reduction in muscle pain (Howatson & van Someren, 2008; Sousa et al. 2014; Urso 2013). Indeed, these are the proposed mechanisms that underpin the effects seen with other functional foods on recovery after muscle-damaging exercise (Howatson et al. 2010; Trombold et al. 2010). Yet, unlike the other functional foods shown to attenuate EIMD, BTJ is unique in that it also contains high amounts of nitrate, a precursor for endogenous nitric oxide (NO) production via the nitrate-nitrite-NO pathway (Lundberg et al. 2008; Kapil et al. 2014). It has become apparent that NO has a regulatory influence on several of the biological processes often impaired (microvascular blood flow,  $\text{Ca}^{2+}$  handling)

(Ferguson et al. 2013; Hernandez et al. 2013; Hoon et al. 2015) or upregulated (phagocytosis, calpain activity and myogenesis) (Jädert et al. 2012; Lomonosova et al. 2014; Rigamonti et al. 2013) after muscle-damaging exercise. Due to the potential involvement of NO in the damage and repair processes in skeletal muscle, it has been suggested that NO donors, such as nitrate for example, could offer a therapeutic approach to enhance recovery after muscle injury inflammation (Lomonosova et al. 2014; Rigamonti et al. 2013). With that said, it is also important to point out that nitrates conversion to NO, and thus its bioavailability, is thought to be facilitated by a low partial pressure oxygen ( $PO_2$ ) and pH within tissues (Lundberg et al. 2008; Jones, 2014), and therefore the levels needed for beneficial physiological effects might not be achievable in healthy tissues; that is muscle tissue with  $PO_2$  and pH within the normal ranges, as would be expected in the days following an exercise bout e.g., at rest. Nonetheless, the effects of nitrate on exercise recovery, independent of BTJ, are still yet to be tested in humans to confirm or refute a potential role in recovery.

Some of the effects mentioned above that have been attributed to NO make the expectation tenable that it could be the main compound in BTJ responsible for exerting the beneficial effects we have recently observed on exercise recovery (Clifford et al. 2016a, c). If this is the case, because of the plethora of biological processes that NO is involved in, mechanisms other than AOX and anti-inflammatory could provide an explanation for some of the beneficial effects we have reported with BTJ on recovery. Furthermore, it would also help to answer the question of whether these effects are exclusive to BTJ and its somewhat unique mixture of phytonutrients, or whether similar effects could be expected with foods that are just simply rich in nitrate. Thus, the aim of this study was to investigate the effects of BTJ versus a nitrate only containing drink (SN) on muscle damage following a bout of eccentric-heavy exercise. It is important to note that we were specifically interested on the effects of nitrate on the functional outcomes of muscle damage (e.g., muscle pain and muscle function loss) and not its effects on oxidative stress, or at the skeletal muscle level, because we were unable to analyse these aspects in the present study. We therefore stress that their mention in the introduction is simply to inform the reader of the potential mechanisms by which we speculate BTJ and or nitrate might benefit recovery. It was hypothesized that BTJ and SN would be similarly effective for attenuating indices of muscle damage compared to a PLA.

## **Materials and Methods**

### *Participants*

According to our previous work (Clifford et al. 2016a), a sample size of  $n = 10$  per group was sufficient to detect an 8% ( $ES = 1.25$ ) between group difference ( $SD: 6\%$ ) in CMJ at a power of 0.80 and  $\alpha$  level of 0.05. Consequently, 30 healthy male participants were recruited to participate in this study (Table 1). All participants were recreationally active and engaged in some form of exercise  $1-3 \text{ d}\cdot\text{wk}^{-1}$  but none had completed intense plyometric exercise for  $>12$  months. Participants provided written informed consent and completed a health screening questionnaire prior to study entry. The use of any dietary supplements (i.e., multivitamins, whey protein and creatine), pain relief medications (i.e., non-steroidal anti-inflammatory drugs) or putative recovery treatments (i.e., compressions garments, massage) was prohibited throughout testing. The study procedures received institutional ethical approval and were conducted in accordance with the Declaration of Helsinki.

### *Experimental design*

The study employed a double blind, randomized, independent groups design. Prior to data collection, participants were required to attend a familiarisation session in which height, body mass and maximal isometric voluntary contractions (MIVC) were established. Using MIVC scores as a blocking factor, participants were then randomized to 1 of 3 experimental treatment groups: SN;  $n=10$ , BTJ;  $n=10$  or PLA;  $n=10$ . At least 1 week after familiarisation participants attended the laboratory on 4 consecutive mornings. On day 1, participants performed a strenuous plyometric exercise protocol to induce muscle damage. A venous blood draw, muscle soreness and measures of muscle function were taken pre and immediately post muscle-damaging exercise on day 1 and on the following 3 mornings (24, 48 and 72 h post-exercise). After the muscle-damaging exercise on day 1, participants consumed 1 serving of their allocated supplement alongside a standardized breakfast meal. Breakfast consisted of cereal (Rice Krispies, Kellogs, UK) and milk (semi-skimmed, Tesco Ltd, UK) and provided 10% of daily energy requirements calculated from age and body mass (kg) (Schofield 1984). Following a 2 h absorption period a final blood draw was taken. Participants could consume water *ad libitum* during the absorption period but were required to avoid consuming any other foods until the final blood draw.

### *Muscle-damaging protocol*

The muscle damaging protocol consisted of 100 drop jumps from a 0.6 m high box and has proved to be an effective means of inducing muscle-damage in several previous studies (Howatson et al. 2009; Clifford et al. 2016a). Briefly, upon landing, participants were required

to descend into a squat ( $\sim 90^\circ$  knee flexion) and then jump vertically with maximal force. Each jump was separated by 10 seconds and each 20 jumps by a 2-minute rest period.

#### *Pressure pain-threshold*

As in several previous studies (Clifford et al. 2016; Bowtell et al. 2010; Connolly et al. 2006), pressure-pain threshold (PPT) was used to assess site-specific muscle soreness. All measures were taken with a handheld algometer (Wagner Instruments, Greenwich CT, US) and with the participant lying supine on a medical bed. The specific muscles tested were: vastus lateralis (mid-way between the superior aspect of the greater trochanter and head of the tibia), rectus femoris (mid-way between the anterior patella and inguinal fold) and gastrocnemius (medial aspect of the calf at relaxed maximum girth). PPT was recorded as the first instance of pain felt when pressure was applied at a constant rate of  $10 \text{ N cm}^{-2} \cdot \text{s}^{-1}$  to the muscle belly. Two recordings were taken and the average used for analysis, unless these values differed by  $10 \text{ N}^2$ , in which case a third measure was taken and the two closest values averaged. The inter-day CV for this procedure was calculated as  $<8\%$  (average CV for the 3 sites measured).

#### *Maximal isometric voluntary contraction*

As in previous studies (Clifford et al. 2016; Howatson et al. 2009), MIVC was measured using a portable strain gauge (MIE Medical Research Ltd., Leeds, UK). In a seated position, participants exerted maximal force against a plinth attached to their right ankle, just above the malleoli. They were instructed to hold this contraction for 3 seconds and were provided strong verbal encouragement for each effort. Three contractions were performed, separated by 60 sec of passive (seated) recovery. The peak value was used for analysis. The coefficient of variation (CV) for this protocol was  $1.1\%$ .

#### *Counter movement jump*

To perform the counter movement jump (CMJ) test, participants descended into a squat (to a  $\sim 90^\circ$  knee angle) and jumped vertically with maximum effort, keeping their hands on hips throughout the entire movement. Participants performed 3 maximal efforts, interspersed by 30 seconds passive (standing) recovery with the mean height of the 3 jumps used for analysis. The CV for measuring CMJ in our lab been was previously calculated as  $<2.5\%$ .

#### *Blood sampling and analysis*

Venous blood samples were obtained pre-exercise, 2 h post-breakfast, 24, 48 and 72 h post exercise via venepuncture. With the exception of the sample taken 2 h after breakfast, all samples were obtained following a  $\geq 12$  h overnight fast. Blood was collected into serum (1x10

ml) vacutainers and subsequently centrifuged at 3000 g (4°) for 15 minutes after ~30 min was allowed for clotting. The serum supernatant was aspirated into a series of aliquots and immediately stored at -80° C for later analysis of creatine kinase (CK), high sensitivity C-reactive protein (hs-CRP) and NO. CK and hs-CRP were measured in serum using an automated electrochemiluminescence method (Roche Modular, Roche Diagnostics, Indianapolis, IN, USA). The laboratory calculated the CV for this analysis to be <2%. Because we did not have the facilities to measure nitrate and nitrite using chemiluminescence or liquid chromatography methods, we elected to estimate the sum total of nitrate and nitrite (NO<sub>x</sub>) in serum using the griess reaction with a commercially available assay kit (R&D Systems, Minneapolis, Minnesota). This assay has been successfully used to quantify NO<sub>x</sub> in a previous study with nitrate supplementation and exercise (Christensen et al., 2013). The CV for this measure was <15%; the sensitivity of this assay is calculated as 0.78 umol/L.

### *Supplementation*

Participants received BTJ, SN or an isocaloric placebo (PLA) drink for 3 days' post muscle-damaging exercise. Table 2 provides an overview of each supplement. A SN drink was used instead of the nitrate-depleted BTJ drink used in other studies (Gilchrist et al. 2013; Muggeridge et al. 2013) because we could not source this from the manufacture. Supplements were consumed on three occasions on day 1; one 30 min post-exercise alongside a breakfast meal, one 2.5 h post-exercise after an additional blood sample, and a third with their evening meal. Participants consumed 2 more servings at 24 and 48 h post (the first within 30 min of completing all dependent variables and the second with their evening meal). SN was purchased in powder form (BASF, Ludwigshafen, Germany) and mixed with water into bottles for participants to consume as a drink. Both maltodextrin (Myprotein, Manchester, UK) and flavourless protein powder (Arla Foods, Amba, Denmark) were added to each serving of SN and PLA to match the BTJ for macronutrient composition. The SN and BTJ were matched as closely as possible for nitrate content. The nitrate content of this particular batch of BTJ was approximately 210 mg (~3.4 mmol/L) per 250 ml serving (data from the manufacturer). This amount of nitrate was equivalent to 287 mg of SN after adjusting for differences in molecular weight; thus, 287 mg (~3.4 mmol/L) of SN powder was carefully weighed and added to each 250 ml serving. All 3 supplements were provided in masked bottles that were identical in size and appearance. The drinks could not be taste-matched and therefore the study aims were concealed from the participants; in other words, they were not informed that BTJ was under investigation, just that they would be drinking newly developed antioxidant-based recovery

drinks. The independent groups design ensured that each group was unaware of what the other drinks under investigation were and thus they were never made aware if their drink was the experimental treatment. We have implemented this strategy in several previous studies (Clifford et al. 2016a; Clifford et al. 2016c).

#### *Dietary control*

Participants were asked to not deviate from their usual eating pattern and record their food and fluid intake throughout the trial using the food diaries provided. However, as in our previous work (Clifford et al. 2016a), they were prohibited from using antibacterial mouthwash throughout data collection due its potential interference with nitrate-nitrite conversion. In addition, prior to each study visit, participants were provided with a meal (Beef Lasagne, 450 g; Tesco Ltd, UK) and a snack bar (Honey and Oat Cunch Bar, 42 g; Natures Valley, UK) to consume as a replacement for their usual evening meal. Participants were instructed to consume both the foods together at least 12 h prior to their study visit the following morning and to avoid consuming any other food or drink (other than water) until all measures had been completed that day. The meal and snack bar provided 836 kcal, of which 34.6% was carbohydrates, 18.9% protein and 43.7% fat.

#### *Data analysis*

All data were analysed using IBM SPSS Statistics 22 for Windows (Surrey, UK) and are presented as mean  $\pm$  SD. Multiple one-way ANOVA's were used to test for group differences between participant's physical characteristics and dietary intake. Food diaries were analysed for macronutrient content using Nutritics dietary analysis software (Nutritics LTD, Dublin, Ireland). CMJ, MIVC and PPT were measured using a mixed design ANOVA; 3 group levels (BTJ vs. SN vs. PLA) by 5 time levels (pre-exercise, post-exercise, 24, 48 and 72 h post-exercise). Data analysis for these measures was performed on values corrected for percentage change from baseline. Biochemical markers were analysed with a 3 (group) x 6 (time; pre-exercise, post-exercise, 2.5 h post-exercise, 24, 48 and 72 h post-exercise) mixed design ANOVA. Significance was read from the Greenhouse-Geisser adjustment if Mauchly's test of Sphericity had been violated. In the event of a significant interaction effect (drink\*time) Fisher LSD *post hoc* analysis was performed to locate where the differences occurred. Where relevant, Cohen's *d* ES were calculated with the magnitude of effects considered small (0.2-0.49), medium (0.5-0.79) and large ( $\geq 0.8$ ). Statistical significance was set at  $P < 0.05$  prior to analyses.

### **Results**



There were no significant differences between groups for their physical characteristics or macronutrient intake throughout the testing period (Table 1;  $P > 0.05$ ). Serum NOx concentrations showed group ( $P = 0.007$ ) and group\*time interaction effects ( $P = 0.004$ ), increasing after BTJ and SN but not PLA. As shown in Figure 1, 2 h after consuming BTJ and SN serum NOx was markedly higher ( $P < 0.001$ ;  $135.5 \pm 78.7$  and  $189.2 \pm 78.8$   $\mu\text{mol/L}$ , respectively) than baseline and PLA concentrations. Serum NOx remained elevated above baseline for the rest of the trial in the BTJ and SN groups ( $P < 0.05$ ).

PPT showed a main effect for both time ( $P = 0.001$ ) and group ( $P = 0.043$ ) whereby PPT was reduced in all groups as a result of exercise but consistently higher in BTJ compared to the SN and PLA in the 72 h post-exercise period (Figure 2). PPT had recovered to baseline values in the BTJ group by 72 h ( $104.3 \pm 25.9\%$ ) but remained depressed in both the SN ( $94.1 \pm 16.0\%$ ) and PLA groups ( $91.2 \pm 19.0\%$ ) (ES = 0.69 vs PLA and 0.53 vs. SN).

There was an immediate decline in MIVC and CMJ following the exercise bout (time effect;  $P < 0.05$ ) with neither variable recovering to baseline by 72 h post-exercise (Table 3). There were no differences between the three groups at any time point for MIVC and CMJ ( $P > 0.05$ ). Serum CK increased in response to the exercise bout (time effect;  $P = 0.011$ ), peaking at 24 h post in all groups (Table 3); however, no group or interaction effects were present ( $P > 0.05$ ). hs-CRP was unaltered following exercise and showed no time, group or interaction effects ( $P > 0.05$ ).

## **Discussion**

The aim of this study was to compare the effects of a nitrate matched BTJ and SN drink on muscle force loss and muscle pain after eccentric exercise, in an attempt to gain a better understanding of the potential effects of nitrate on EIMD. While ingestion of BTJ and SN increased serum NOx levels, neither drink protected against muscle force deficits (MIVC and CMJ) in the 72 h after the exercise bout. However, BTJ was more efficacious for attenuating muscle pain than SN and a PLA. A biochemical marker of muscle damage (CK) and a general marker of inflammation (hs-CRP) did not differ between supplement groups at any time point. As expected, provision of BTJ and SN after muscle-damaging exercise evoked large increases in serum NOx concentrations compared to PLA, 2.5 h post (Figure 2). These results are in agreement with the findings from our previous work (Clifford et al. 2016b) and others (Cristensen et al. 2012; Joris and Menesik 2013) who observed large increases in circulating NOx bioavailability after SN and BTJ ingestion. In the subsequent days, the NOx levels were much lower, likely due to the fact the last dose was provided ~12 h before being measured and

therefore samples were not taken at peak concentrations on these occasions. Importantly, the rise in serum NO<sub>x</sub> was similar between SN and BTJ at 2.5 h post ingestion, indicating that the drinks evoked similar levels of NO<sub>x</sub> and were therefore well-matched for nitrate content.

Recent data have shown that NO is integral for normal muscle regeneration, and that administering NO donors enhances the recovery of strength after eccentric exercise (Lomonsova et al. 2014; Rigamonti et al. 2013). Nevertheless, the present findings are in contrast to the above data, instead suggesting that SN supplementation is ineffective for attenuating force loss or any other parameter of EIMD after an eccentric-heavy exercise bout. The most obvious explanation for the discrepant findings between the present and aforementioned studies is the species difference; the present study was in humans, whereas most previous studies suggesting a role for NO in EIMD were in animals (Corona and Ingalls, 2013; Lomonsova et al. 2014; Rigamonti et al. 2013; Sakurai et al., 2013). However, it is also possible that other methodological differences, such as exercise model, NO donor and dosage contributed to the discrepancies. Additionally, it could also be due to the fact that, as alluded to in the introduction, nitrate to NO conversion was not sufficient in the tissues because preferable conditions for this conversion were not met (e.g., ischemia, low PO<sub>2</sub> and pH) (Jones, 2014). Notwithstanding, the fact that SN had no influence on any of the indices of muscle damage measured in this study, would seem to indicate that nitrate, at least at this dose, might not exert any favourable effects on exercise recovery. These findings also suggest that nitrate might not be the main constituent in BTJ responsible for attenuating losses in muscle function in previous studies (Clifford et al. 2016a, c).

The inability of BTJ to attenuate the post-exercise loss in CMJ height is in direct contrast to a previous investigation (Clifford et al., 2016a), in which 48 and 72 h after the same bout of plyometric exercise, BTJ was found to attenuate the deficit in CMJ. Because in both studies muscle damage was induced with an identical protocol, and the participants were similar in terms of training status, this discrepancy is difficult to account for. Nevertheless, a possible explanation could lie in the differences in dietary control between the two investigations. In the previous study, a strict low-phytonutrient diet was imposed 48 h prior to and throughout the course of the trial (5 days in total), whereas in the present study, participants were encouraged to not deviate from their usual eating patterns. This change in design was to ensure the findings of the present study were more ecologically valid and applicable to real-world scenarios, in which individuals, particularly athletes, do not restrict their phytonutrient intake. Nonetheless, it has been demonstrated that restricting the intake of AOX and phytonutrient rich

foods in the diet can leave individuals more vulnerable to oxidative stress (Watson et al., 2005) and inflammation (Plunkett et al. 2009) after stressful exercise. This could have important implications for secondary muscle damage after strenuous exercise, and muscle function (Paschalis et al. 2016), and makes the expectation tenable that dietary intake of AOX and/or phytonutrient rich foods could influence an individual's susceptibility to EIMD, and, thus, their ability to recover from muscle-damaging exercise.

Interestingly, the CMJ loss in response to the drop jumps in our previous study was clearly greater than in the present study, which would lend some support to the idea that a low phytonutrient intake might impact an individual's rate of recovery. Indeed, the recovery of CMJ performance was more prolonged in the 72 h following the drop jumps in that study, and there was a clear secondary loss in muscle function without BTJ supplementation in the 24-72 h after exercise, which was also less pronounced in the present study. Just to illustrate, CMJ height was -25% and -14% at 48 and 72 h post-exercise in the PLA group in our previous work (Clifford et al. 2016a), compared to -14% and -8% in the present study. These results provide tentative support for the idea that restricting phytonutrients through the diet might leave an individual more vulnerable to muscle damage, at least after eccentric-heavy exercise, in which secondary damage is presumed to be of a higher magnitude and more prolonged (Howatson and van Someren, 2008). If this is the case, it would be reasonable to assume that BTJ would be more beneficial for functional recovery and performance in individuals with lower AOX intakes and/or higher oxidative stress, as has been recently suggested with vitamin C supplementation (Paschalis et al. 2016). This might help to explain, at least in part, why BTJ attenuated the loss in CMJ in our previous work, when phytonutrient intake was restricted (and participants might have been more susceptible to EIMD) but not in present study, when phytonutrient intake was unrestricted (thus participants were possibly less vulnerable to EIMD). Clearly, without measuring phytonutrient intake and inflammation/oxidative stress (and other aspects that might influence secondary muscle damage) in either of these studies the above postulate is speculative, and needs to be clarified in future work. We acknowledge that not exploring oxidative stress or inflammation in the present study is a limitation and therefore interpretation of the mechanisms behind these findings need to be treated with caution, especially as we pointed out in the introduction, data to support an AOX effect of BTJ or nitrate remains equivocal.

As in previous studies, serum CK efflux increased after exercise, irrespective of supplementation. These data support the conclusions of others who have also found that

functional foods do not seem to reduce CK efflux to a greater extent than a PLA (Bell et al. 2014; Bell et al. 2015; Goldfarb et al. 2011; Howatson et al. 2010; Peschek et al. 2013). It is important to note that CK is not considered a valid enough measure for assessing the extent of EIMD (Paulsen et al. 2012; Warren et al. 1999) and, thus, it might not be a sensitive enough marker to detect changes associated with an intervention. Hence, the primary outcome measures were changes in muscle function, which are proposed as the most sensitive and valid markers of EIMD.

Unlike the muscle function measures, muscle pain, as measured by changes in PPT, was alleviated by BTJ supplementation (Figure 2). This pattern for improved recovery of PPT is consistent with our previous work, where PPT recovered quicker with BTJ versus a PLA after eccentric-heavy exercise. An explanation as to how BTJ might reduce muscle pain is still unclear. Part of the difficulty in determining the potential mechanisms involved stems from the fact that the precise causes of exercise-induced muscle pain are still uncertain (Yu et al. 2013). Nonetheless, as described before, a possibility is that BTJ might suppress the release of stimuli thought to sensitize nociceptive neurons in the muscle and ECM (Murase et al. 2013), the latter of which, to the best of the current knowledge, is proposed as the main site where muscle pain originates (Crameri et al. 2007). However, because muscle tissue samples could not be obtained in this study, this posit is somewhat speculative until confirmed by future studies; we acknowledge that our inability to collect muscle tissue and investigate these mechanisms is a limitation of this study.

Perhaps the most pertinent new finding of this study is that muscle pain was attenuated with BTJ but not SN. These results suggest that phytonutrients other than nitrate, such as betalains and phenolics, or interactions between them (or with nitrate), are likely responsible for its analgesic effects. Although there have been no previous attempts to directly compare the effects of nitrate and BTJ on muscle pain, reports that nitrate free but betalain-rich treatments can alleviate muscle pain appear to support this concept. In two studies (Pietrzkowski et al. 2010 & Pietrzkowski et al. 2014) muscle and joint pain, as measured with the McGill pain questionnaire, was significantly lower after 10 days of taking specially formulated betalain capsules derived from beetroot extracts. The supplements used in these studies did not contain any of the nitrate or phenolic compounds inherently found in BTJ, therefore suggesting that betalains, independent of any interactions with other biological compounds, were responsible for alleviating muscle and joint pain. Based on these findings, the improved PPT seen in the present study with BTJ might have been due to the high amount of betalains it contains,

particularly betanin (see Table 2). With that said, it is important to point out, that because we were unable to measure the potential mechanisms underpinning these findings, the pain alleviating effects could simply be an artefact of the subjective nature of measuring muscle pain, and not due to a true physiological effect—especially as this was not a crossover study and therefore the participants did not rate pain in response to the different treatments. However, when these findings are coupled with those from the previously aforementioned studies (Pietrzkowski et al. 2010 & Pietrzkowski et al. 2014) and our own (Clifford et al. 2016a; Clifford et al. 2016c) which demonstrated a pain-relieving effect with beetroot or its individual constituents, we are more confident that the findings of this study are due to a true physiological effect. Yet, we stress that further studies are still needed to support these findings.

In conclusion, this study found that acute supplementation with BTJ and SN did not influence the recovery of muscle function or attenuate CK efflux or hs-CRP after exercise. BTJ was more beneficial than SN for attenuating PPT though, advocating the use of BTJ over nitrate only containing drinks for attenuating exercise-induced muscle pain. Relief from exercise-induced muscle pain is desirable given the fact that muscle pain can alter movement patterns and heighten injury risk (Hodges & Tucker, 2011; Cheung et al. 2003); thus, BTJ might be useful to those regularly taking part in sport or strenuous physical activity. These findings also suggest that the phenolics and betalains could be the compounds in BTJ most likely to exhibit analgesic effects after exercise and therefore further exploration of their potential for pain relief is warranted—not only in exercise but perhaps also clinical settings.

### **Conflict of interest**

This study was funded as part of a doctoral degree that receives financial support from Gs Fresh Ltd. The funders supplied the supplements used in this study but had no role in the conception of the study, its design, preparation, analysis and writing of the manuscript. The authors declare no conflict of interest.

### **Acknowledgements**

The authors wish to thank Northumbria University for part funding this study. This is contribution number 188 from the NWU-Water Research Group.

## **Reference list**

- Ashor, A. 2016. Inorganic nitrate does not affect glucose and insulin regulation but lowers oxidative stress in obese adults during acute hyperglycaemia. *Atherosclerosis*: 252, e94.
- Bell, P.G., Walshe, I.H., Davison, G.W., Stevenson, E., and Howatson, G. 2014. Montmorency cherries reduce the oxidative stress and inflammatory responses to repeated days high-intensity stochastic cycling. *Nutrients* 6(2): 829-43.
- Bell, P.G., Walshe, I.H., Davison, G.W., Stevenson, E.J., and Howatson, G. 2015. Recovery facilitation with Montmorency cherries following high-intensity, metabolically challenging exercise. *Appl Physiol Nutr Metab* 40(4): 414-23.
- Bowtell, J.L., Sumners, D.P., Dyer, A., Fox, P., and Mileva, K.N. 2011. Montmorency cherry juice reduces muscle damage caused by intensive strength exercise. *Med Sci Sports Exerc* 43(8): 1544-51.
- Cheung, K., Hume, P. A., & Maxwell, L. 2003. Delayed onset muscle soreness. *Sports Med*, 33(2): 145-164.
- Christensen, P., Nyberg, M., & Bangsbo, J. 2012. Influence of nitrate supplementation on VO<sub>2</sub> kinetics and endurance of elite cyclists. *Scandinavian Journal of Medicine and Science in Sports* 23(1): e21-e31.
- Clifford, T., Bell, O., West, D.J., Howatson, G., and Stevenson, E.J. 2016a. The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise. *Eur J Appl Physiol* 116(2): 353-62.
- Clifford, T., Constantinou, C.M., Keane, K.M., West, D.J., Howatson, G., and Stevenson, E.J. 2016b. The plasma bioavailability of nitrate and betanin from *Beta vulgaris rubra* in humans. *Eur J Nutr*. DOI: 10.1007/s00394-016-1173-5.
- Clifford, T., Berntzen, B., Davison, G.W., West, D.J., Howatson, G. and Stevenson, E.J., 2016c. Effects of beetroot juice on recovery of muscle function and performance between bouts of repeated sprint exercise. *Nutrients* 8(8): 506-520.
- Clifford, T., Allerton, D.M., Brown, M.A., Harper, L., Horsburgh, S., Keane, K.M., Stevenson, E.J. and Howatson, G., 2016d. Minimal muscle damage after a marathon and no influence of beetroot juice on inflammation and recovery. *Appl Physiol Nutr Met*, (ja). DOI: 10.1139/apnm-2016-0525.

- Clifford, T., Howatson, G., West, D.J. and Stevenson, E.J., 2015. The potential benefits of red beetroot supplementation in health and disease. *Nutrients* 7(4): 2801-2822.
- Connolly, D. A., McHugh, M. P., Padilla-Zakour, O. I., Carlson, L., & Sayers, S. P. 2006. Efficacy of a tart cherry juice blend in preventing the symptoms of muscle damage. *British Journal of Sports Med* 40(8): 679-683.
- Corona, B.T. and Ingalls, C.P., 2013. Immediate force loss after eccentric contractions is increased with l- name administration, a nitric oxide synthase inhibitor. *Muscle & Nerve*, 47(2), pp.271-273.
- Crameri, R. M., Aagaard, P., Qvortrup, K., Langberg, H., Olesen, J., & Kjaer, M. 2007. Myofibre damage in human skeletal muscle: effects of electrical stimulation versus voluntary contraction. *J Physiol* 583(Pt 1): 365-380.
- El Gamal, A.A., AlSaid, M.S., Raish, M., Al-Sohaibani, M., Al-Massarani, S.M., Ahmad, A., Hefnawy, M., Al-Yahya, M., Basoudan, O.A., and Rafatullah, S. 2014. Beetroot (*Beta vulgaris* L.) extract ameliorates gentamicin-induced nephrotoxicity associated oxidative stress, inflammation, and apoptosis in rodent model. *Mediators Inflamm* 2014: 983-952.
- Ferguson, S. K., Hirai, D. M., Copp, S. W., Holdsworth, C. T., Allen, J. D., Jones, A. M., ... Poole, D. C. 2012. Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *J Physiol* 591(2): 547–557.
- Goldfarb, A. H., Garten, R. S., Cho, C., Chee, P. D., & Chambers, L. A. 2011. Effects of a fruit/berry/vegetable supplement on muscle function and oxidative stress. *Med Sci Sports Exerc*, 43(3): 501-508.
- Hernández, A., Schiffer, T. A., Ivarsson, N., Cheng, A. J., Bruton, J. D., Lundberg, J. O., ... Westerblad, H. 2012. Dietary nitrate increases tetanic  $[Ca^{2+}]$  and contractile force in mouse fast-twitch muscle. *J Physiol* 590(15): 3575–3583.
- Hodges, P. W., & Tucker, K. 2011. Moving differently in pain: a new theory to explain the adaptation to pain. *Pain*, 152(3): S90-S98.
- Hoon, M. W., Fornusek, C., Chapman, P. G., & Johnson, N. A. 2015. The effect of nitrate supplementation on muscle contraction in healthy adults. *Eur J Sport Sci* 15(8): 712–719.
- Howatson, G. and van Someren, K.A. 2008. The prevention and treatment of exercise-induced muscle damage. *Sports Med* 38(6): 483-503.
- Howatson, G., Goodall, S., & van Someren, K. A. 2009. The influence of cold water immersions on adaptation following a single bout of damaging exercise. *Eur J Appl Physiol* 105(4): 615-621.

Howatson, G., McHugh, M.P., Hill, J.A., Brouner, J., Jewell, A.P., van Someren, K.A., Shave, R.E., and Howatson, S.A. 2010. Influence of tart cherry juice on indices of recovery following marathon running. *Scand J Med Sci Sports* 20(6): 843-52.

Jadert, C., Petersson, J., Massena, S., Ahl, D., Grapensparr, L., Holm, L., Lundberg, J.O., and Phillipson, M. 2012. Decreased leukocyte recruitment by inorganic nitrate and nitrite in microvascular inflammation and NSAID-induced intestinal injury. *Free Radic Biol Med* 52(3): 683-92.

Jones, A. M. 2014. Dietary nitrate supplementation and exercise performance. *Sports Med*, 44(1): 35-45.

Joris, P. J., & Mensink, R. P. 2013. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. *Atherosclerosis* 231(1): 78-83.

Justice, J. N., Gioscia-Ryan, R. A., Johnson, L. C., Battson, M. L., de Picciotto, N. E., Beck, H. J., ... & Seals, D. R. 2015. Sodium nitrite supplementation improves motor function and skeletal muscle inflammatory profile in old male mice. *J Appl Physiol* 118(2): 163-169.

Kapil, V., Weitzberg, E., Lundberg, J., & Ahluwalia, A. 2014. Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health. *Nitric Oxide* 38: 45-57.

Larsen, F. J., Schiffer, T. A., Ekblom, B., Mattsson, M. P., Checa, A., Wheelock, C. E., ... & Weitzberg, E. 2014. Dietary nitrate reduces resting metabolic rate: a randomized, crossover study in humans. *The American Journal of Clinical Nutrition*, 99(4): 843-850.

Lomonosova, Y. N., Shenkman, B. S., Kalamkarov, G. R., Kostrominova, T. Y., & Nemirovskaya, T. L. 2014. L-arginine supplementation protects exercise performance and structural integrity of muscle fibres after a single bout of eccentric exercise in rats. *PloS One* 9(4): 944-948.

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. 2008. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery* 7(2): 156-167.

Murase, S., Terazawa, E., Hirate, K., Yamanaka, H., Kanda, H., Noguchi, K., Ota, H., Queme, F., Taguchi, T., and Mizumura, K. 2013. Upregulated glial cell line-derived neurotrophic factor through cyclooxygenase-2 activation in the muscle is required for mechanical hyperalgesia after exercise in rats. *J Physiol* 591(12): 3035-48.

Paschalis, V., Theodorou, A., Kyparos, A., Dipla, K., Zafeiridis, A., & Panayiotou, G. et al. 2016. Low vitamin C values are linked with decreased physical performance and increased oxidative stress: reversal by vitamin C supplementation. *Eur J Nutr*, 55(1): 45-53.



- Paulsen, G., Mikkelsen, U. R., Raastad, T., & Peake, J. M. 2012. Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? *Exercise Immunology Review* 18: 42-97.
- Peschek, K., Pritchett, R., Bergman, E., & Pritchett, K. 2013. The effects of acute post exercise consumption of two cocoa-based beverages with varying flavanol content on indices of muscle recovery following downhill treadmill running. *Nutrients* 6(1): 50-62.
- Pietrzkowski Z, N.B., Spórna A, Stalica P, Tresher W, Keller R, Jimenez R, Michałowski T, Wybraniec S. *New Medicine*. 2010. Influence of betalain-rich extract on reduction of discomfort associated with osteoarthritis *New Med* 1: 112-17.
- Pietrzkowski, Z., Argumedo, R., Shu, C., Nemzer, B., Wybraniec, S., & Reyes-Izquierdo, T. 2014. Betalain-rich red beet concentrate improves reduced knee discomfort and joint function: a double blind, placebo-controlled pilot clinical study. *Nutr Diet Sup* 6: S59042.
- Plunkett, B. A., Callister, R., Watson, T. A., & Garg, M. L. 2010. Dietary antioxidant restriction affects the inflammatory response in athletes. *Brit J Nutr* 103(08): 1179-1184.
- Rigamonti, E., Touvier, T., Clementi, E., Manfredi, A. A., Brunelli, S., & Rovere-Querini, P. 2013. Requirement of inducible nitric oxide synthase for skeletal muscle regeneration after acute damage. *J Immunol* 190(4): 1767-1777.
- Schofield, W. N. (1984). Predicting basal metabolic rate, new standards and review of previous work. *Human nutrition. Clinical nutrition*, 39: 5-41.
- Sousa, M., Teixeira, V.H., and Soares, J. 2014. Dietary strategies to recover from exercise-induced muscle damage. *Int J Food Sci Nutr* 65(2): 151-63.
- Trombold, J.R., Barnes, J.N., Critchley, L., and Coyle, E.F. 2010. Ellagitannin consumption improves strength recovery 2-3 d after eccentric exercise. *Med Sci Sports Exerc* 42(3): 493-8.
- Trombold, J.R., Reinfeld, A.S., Casler, J.R., and Coyle, E.F. 2011. The effect of pomegranate juice supplementation on strength and soreness after eccentric exercise. *J Strength Cond Res* 25(7): 1782-8.
- Urso, M.L. 2013. Anti-inflammatory interventions and skeletal muscle injury: benefit or detriment? *J Appl Physiol* (1985) 115(6): 920-8.
- Vidal, P.J., Lopez-Nicolas, J.M., Gandia-Herrero, F., and Garcia-Carmona, F. 2014. Inactivation of lipoxygenase and cyclooxygenase by natural betalains and semi-synthetic analogues. *Food Chem* 154: 246-54.
- Warren, G. L., Lowe, D. A., & Armstrong, R. B. 1999. Measurement tools used in the study of eccentric contraction-induced injury. *Sports Medicine*, 27(1), 43-59.

Whitfield, J., Ludzki, A., Heigenhauser, G. J. F., Senden, J. M. G., Verdijk, L. B., Loon, L. J. C., ... & Holloway, G. P. 2016. Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. *J Phys*, 594(2): 421-435.

Watson, T. A., Callister, R., Taylor, R. D., Sibbritt, D. W., MacDonald-Wicks, L. K., & Garg, M. L. 2005. Antioxidant restriction and oxidative stress in short-duration exhaustive exercise. *Medicine and Science in Sports and Exercise*, 37(1): 63-71.

Yu, J., Liu, J., Carlsson, L., Thornell, L., & Stål, P. 2013. Re-Evaluation of Sarcolemma Injury and Muscle Swelling in Human Skeletal Muscles after Eccentric Exercise. *Plos ONE*, 8(4): 620-656.

Table 1. Participant's physical characteristics and macronutrient content of their dietary intake throughout the study.

| <b>Characteristic</b>             | <b>Group</b> |            |            |
|-----------------------------------|--------------|------------|------------|
|                                   | <b>BTJ</b>   | <b>SN</b>  | <b>PLA</b> |
| <b>Age (years)</b>                | 22.6±2.8     | 21.7±2.8   | 21.0±1.4   |
| <b>Mass (kg)</b>                  | 76.7±12.1    | 76.0±7.5   | 72.8±10.8  |
| <b>Height (cm)</b>                | 178.9±7.5    | 179.2±6.9  | 176.1±5.0  |
| <b>Daily energy intake (kcal)</b> | 2460 ± 494   | 2491 ± 706 | 2108 ± 387 |
| <b>Cho (%)</b>                    | 42           | 44         | 43         |
| <b>Pro (%)</b>                    | 18           | 22         | 20         |
| <b>Fat (%)</b>                    | 40           | 34         | 37         |

Values are means ± SD; *n*=10 per group. Groups were not significantly different for any variable (*P* > 0.05). Cho = carbohydrate and Pro = protein.

**Table 2.** Macronutrient and nitrate content of the 3 supplements.

| <b>Supplement</b>                     | <b>BTJ</b> | <b>SN</b> | <b>PLA</b> |
|---------------------------------------|------------|-----------|------------|
| <b>Energy (Kcal)</b>                  | 81.0       | 78.6      | 76.8       |
| <b>Volume (ml)</b>                    | 250        | 250       | 250        |
| <b>Carbohydrate (g)</b>               | 16.4       | 16.4      | 16.4       |
| <b>Protein (g)</b>                    | 2.8        | 2.8       | 2.8        |
| <b>Fat (g)</b>                        | 0.4        | 0.2       | Trace      |
| <b>Nitrate (mg)</b>                   | ~210       | ~210      | N/A        |
| <b>Betanin (mg/L)*</b>                | ~194       | N/A       | N/A        |
| <b>Polyphenol content (mg/GAE/L)*</b> | ~405       | N/A       | ~43        |
| <b>TEAC (mmol/L)*</b>                 | ~3         | N/A       | <0.5       |

GAE, Gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity. \*Based on data from BTJ analysed in Clifford et al. (2016b). The BTJ was provided by the same manufacturer but a different batched was used so a slight variation in these values is likely.

Table 3 - MIVC, CMJ, CK and hs-CRP values pre and post muscle damaging exercise.

| Variable                           | Pre exercise       | Post exercise      | 2.5 h post exercise | 24 h post exercise | 48 h post exercise | 72 h post exercise |
|------------------------------------|--------------------|--------------------|---------------------|--------------------|--------------------|--------------------|
| <b>MIVC (N)*#</b>                  |                    |                    |                     |                    |                    |                    |
| <b>BTJ</b>                         | 602(100) ± 109(0)  | 479(80) ± 144(16)  |                     | 487(81) ± 159(19)  | 510(87) ± 162(10)  | 556(95) ± 164(8)   |
| <b>SN</b>                          | 577(100) ± 100(0)  | 503(87) ± 136(9)   |                     | 510(88) ± 126(10)  | 501(85) ± 151(19)  | 546(92) ± 149(17)  |
| <b>PLA</b>                         | 597(100) ± 117(0)  | 504(84) ± 111(8)   |                     | 505(84) ± 153(15)  | 521(87) ± 136(13)  | 536(89) ± 131(10)  |
| <b>CMJ (cm)*#</b>                  |                    |                    |                     |                    |                    |                    |
| <b>BTJ</b>                         | 31.8(100) ± 7.0(0) | 29.1(90) ± 8.8(11) |                     | 29.2(91) ± 8.4(15) | 29.4(91) ± 8.9(15) | 30.9(97) ± 8.0(10) |
| <b>SN</b>                          | 33.9(100) ± 6.2(0) | 30.1(88) ± 7.0(9)  |                     | 30.5(90) ± 5.5(7)  | 29.7(89) ± 5.2(11) | 32.8(96) ± 5.3(7)  |
| <b>PLA</b>                         | 36.5(100) ± 4.8(0) | 32.0(90) ± 5.2(11) |                     | 32.1(87) ± 6.7(14) | 31.6(86) ± 6.9(15) | 33.7(92) ± 5.6(10) |
| <b>CK (IU·L<sup>-1</sup>)*</b>     |                    |                    |                     |                    |                    |                    |
| <b>BTJ</b>                         | 224 ± 104          | 242 ± 129          | 358 ± 142           | 714 ± 638          | 564 ± 615          | 351 ± 396          |
| <b>SN</b>                          | 198 ± 108          | 229 ± 121          | 264 ± 98            | 312 ± 122          | 192 ± 43           | 148 ± 31           |
| <b>PLA</b>                         | 224 ± 88           | 266 ± 97           | 298 ± 122           | 395 ± 245          | 274 ± 194          | 261 ± 126          |
| <b>hs-CRP (mg·L<sup>-1</sup>)*</b> |                    |                    |                     |                    |                    |                    |
| <b>BTJ</b>                         | 0.41 ± 0.28        | 0.43 ± 0.33        | 0.40 ± 0.29         | 0.35 ± 0.14        | 0.44 ± 0.12        | 0.58 ± 0.82        |
| <b>SN</b>                          | 0.42 ± 0.30        | 0.45 ± 0.30        | 0.42 ± 0.27         | 0.50 ± 0.28        | 0.46 ± 0.26        | 0.39 ± 0.18        |
| <b>PLA</b>                         | 0.44 ± 0.32        | 0.48 ± 0.34        | 0.42 ± 0.31         | 0.42 ± 0.29        | 0.34 ± 0.34        | 0.32 ± 0.13        |

#Values in brackets represents data normalised to percentage change from baseline. \*Time effect; P < 0.05.

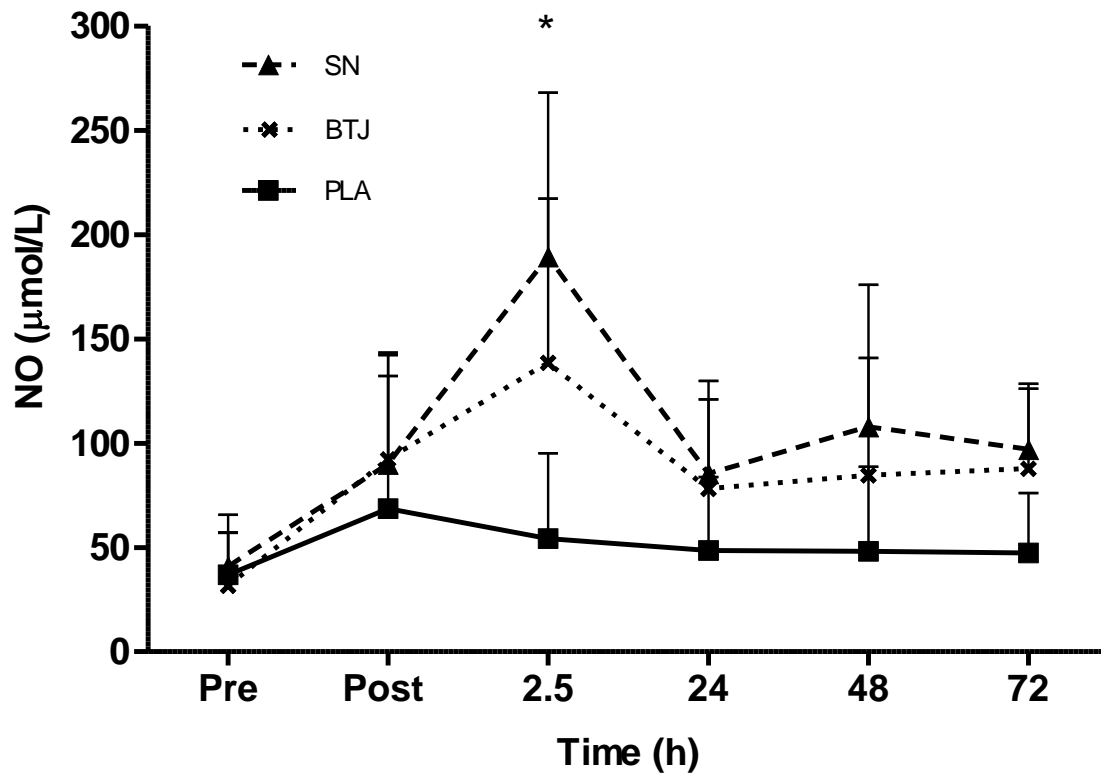


Figure 1 - Serum nitric oxide (NO) concentrations before, immediately post, 2.5 h post breakfast and at selected intervals up to 72 h after exercise. \*Interaction effect; beetroot juice (BTJ) and sodium nitrate (SN) higher than placebo (PLA);  $P < 0.05$ . Data are mean  $\pm$  SD,  $n = 10$  per group.

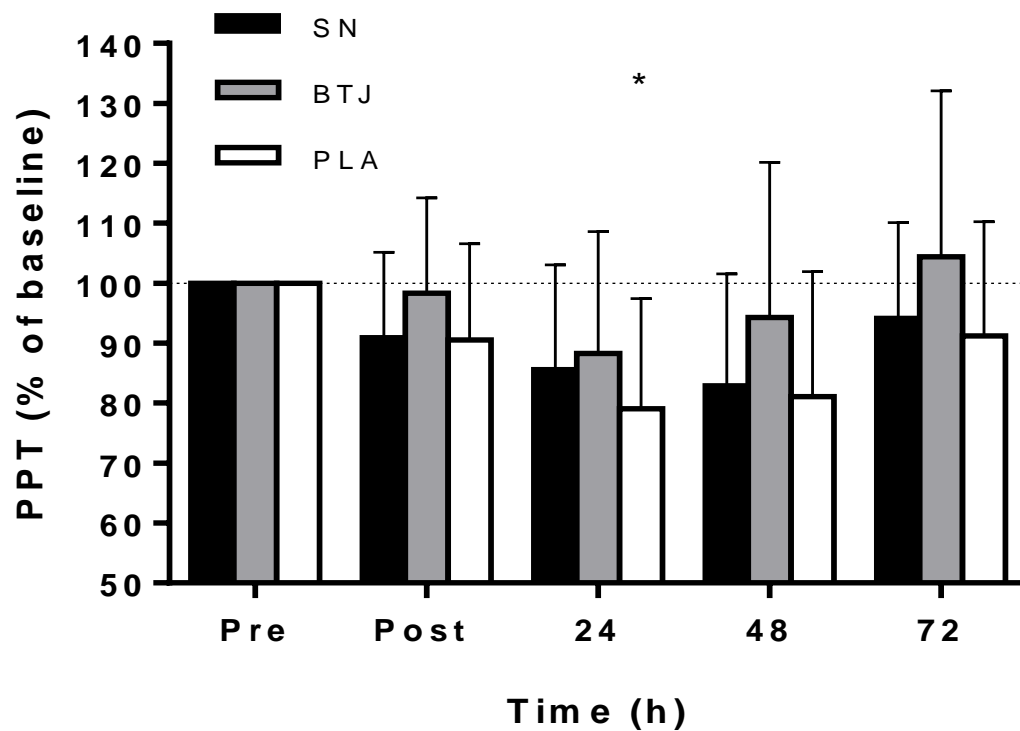


Figure 2 - Pressure pain threshold (PPT) before and 72 h after exercise (% of baseline). Values presented are average of the three sites measured (calf, CF; rectus femoris, RF; vastus lateralis, VL). \*Denotes group effect across all time points post-exercise;  $P < 0.05$ , beetroot juice (BTJ) higher than sodium nitrate (SN) and placebo (PLA). Values are mean  $\pm$  SD;  $n = 10$  per group.